

# HOST-RELATED VARIABILITY IN FATTY ACID COMPOSITION OF SYMBIOTIC CYANOBACTERIA ISOLATED FROM THE AQUATIC FERN, *AZOLLA*

RODULIO CAUDALES<sup>1</sup>, JOHN M. WELLS<sup>2</sup>, JAMES E. BUTTERFIELD<sup>2</sup> and  
ALAN D. ANTOINE<sup>1</sup>

<sup>1</sup>Department of Biochemistry and Microbiology, Rutgers-The State University,  
New Brunswick, NJ, 08903-0231, USA

<sup>2</sup>U.S. Department of Agriculture, ARS, 600 East Mermaid Lane, Philadelphia, PA, 19118,  
USA.

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## INTRODUCTION

Cyanobacteria are a diverse group of oxygenic, photosynthetic prokaryotes of world-wide distribution in marine, aquatic and terrestrial environments (3). Many genera of cyanobacteria are symbiotically associated with a diverse group of plants, lichens, animals and bacteria. *Anabaena* and *Nostoc* are the major genera of cyanobionts found in plants (7).

*Azolla* is an aquatic fern that hosts cyanobacteria in leaf cavities where they fix atmospheric N<sub>2</sub> (4). Traditionally, cyanobionts in *Azolla* have been considered a unique species of *Anabaena azollae*. Recently, Meeks et al. (5) and Plazinski et al. (6) found that cyanobionts are more closely related to the genus *Nostoc* than to *Anabaena*. Caudales and Wells (1) found that cellular fatty acid profiles, determined by gas-liquid chromatography-mass spectroscopy (GLC-MS), provide a rapid and reliable method to differentiate *Anabaena* from *Nostoc*. They also compared fatty acid profiles of *Azolla* cyanobionts with those of *Anabaena* and *Nostoc* and noted 19 individual fatty acids, class totals and ratios that were statistically different in the cyanobionts (2). The data suggested that cyanobionts in *Azolla* may constitute a group or taxa different from *Anabaena* and *Nostoc*.

In the present report we analyze host-related variability in fatty acid composition of symbiotic cyanobacteria isolated from the aquatic fern, *Azolla*.

## MATERIALS AND METHODS

### Media and growth conditions

*Azolla* accessions were grown in defined mineral medium without nitrogen (2). Photon flux density was  $100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  from cool-white fluorescent and incandescent light, using a 14/10 hour light/dark cycle. Temperature was  $23 \pm 1^\circ\text{C}$  during the light and  $16 \pm 1^\circ\text{C}$  during the dark periods. Names of species, strains, geographical origins, and sources of accessions are listed in Table 1.

Cyanobionts from 7 different species of *Azolla* (3 isolates per species) were harvested from 14-18 day old plants as previously described (2). Cyanobacteria were saponified, esterified and then analysed for fatty acids by GLC-MS as previously published (1, 2).

### Statistical analysis

Statistical differences in percentages of selected fatty acids were determined by analysis of variance and Duncan's Multiple Range Test (8). Differences were considered significant at the 95% level of probability ( $P=0.05$ ). Similarity coefficients (S) were calculated for each isolate, based on percentage deviations of the total fatty acid profile from that of the group mean composed of the 21 isolates. Isolates were ranked from 1 to 21 according to greatest similarity to the group mean, and mean values determined for rank and S of isolates from each *Azolla* species.

## RESULTS

Sixty-one different fatty acids were identified, at percent concentrations consistent with those of previously published reports. Seventeen major fatty acid components (each constituting over 0.9% of the total) accounted for 84.31% of the total: the saturated 14:0 (0.98%), 16:0 (34.83%) and 18:0 (1.94%) fatty acids; the unsaturated straight-chained 12:1 (1.16%), 14:1 *cis*-9 (2.03%), 16:1 *cis*-7 (1.04%), 16:1 *cis*-9 (5.30%), 16:1 *cis*-11 (1.96%), 16:3 *cis*-6 (2.35%), 16:4 *cis*-4 (1.04%) 18:2 *cis*-9 (9.45%), 18:3 *cis*-9 (17.88%), 18:1 *cis*-9 (3.06%), 18:1 *trans*-9 (2.03%), and 20:4 *cis*-5 (1.04%); and the branch-chained *iso*-16:0 (2.09%) (Table 2). Also included was an incompletely characterized unsaturated 16-carbon (1.35%) of ECL 15.5. The most abundant component in the group was the 16:0, ranging between 30.29 and 39.53%.

**TABLE 1.** Species and origin of *Azolla* host plants used in this study, and strain designations of their symbiotic cyanobacteria.

| Species and strain | Original strain designation | Geographical origin                             | Source* |
|--------------------|-----------------------------|---|---------|
| A. caroliniana     |                             |   |         |
| C3                 | 3014                        | Rio Negro, Catalao, Amazonas, Brazil            | W.Z.    |
| C2                 | 3010                        | Rio Salimoes, Paciencia Island, Amazonas Brazil | W.Z.    |
| C8                 |                             | Rio Chavon, Dominican Republic                  | R.C.    |

**TABLE 1. Continued** Species and origin of *Azolla* host plants used in this study, and strain designations of their symbiotic cyanobacteria.

| Species and strain          | Original strain designation | Geographical origin           | Source* |
|-----------------------------|-----------------------------|-------------------------------|---------|
| <i>A. Filiculoides</i>      |                             |                               |         |
| F10                         | 103FI                       | Hidalgo, Mexico               | V.H.    |
| F12                         | 126FI                       | Karoo, South Africa           | V.H.    |
| F18                         | 185FI                       | Montevideo, Uruguay           | V.H.    |
| <i>A. mexicana</i>          |                             |                               |         |
| X1                          | X1                          | California, USA (BR-S-CF)     | G.P.    |
| X5                          | 54                          | Guyana 1                      | W.R.    |
| X10                         |                             | Illinois, USA                 | R.C.    |
| <i>A. microphylla</i>       |                             |                               |         |
| M1                          | 65MI                        | Paraiso do Norte, Brazil      | V.H.    |
| M2                          | M2                          | Galapagos, Ecuador (TL-GI-CF) | G.P.    |
| M4                          | 104MI                       | Sinaloa, Mexico               | V.H.    |
| <i>A. nilotica</i>          |                             |                               |         |
| N1                          | 82NI/N1/5001                | Kosti, Sudan                  | VH/GPWZ |
| N2                          | 15NI                        | Burundi                       | V.H.    |
| <i>A. pinnata imbricata</i> |                             |                               |         |
| I1                          | I1                          | Shoolong, China (K2B-T-CF)    | G.P.    |
| I2                          | TDP15                       | Bankok, Thailand              | W.Z.    |
| <i>A. pinnata pinnata</i>   |                             |                               |         |
| P1                          | P1                          | Malaysia (BR-M-CF)            | G.P.    |
| <i>A. rubra</i>             |                             |                               |         |
| R1                          | 163RU                       | Victoria, Australia           | V.H.    |
| R3                          | 204RU                       | Nouvelle, New Zealand         | V.H.    |
| R4                          | 200RU                       | South Island, New Zealand     | V.H.    |

\*. Sources: G.P. = Dr. Gerald Peters, Virginia Commonwealth University, Virginia, USA; W.Z. = Dr. William Zimmerman, University of Michigan, Dearborn, MI, USA; V.H. = Dr. Charles Van Hove, Universite Catholique de Louvain at Louvain-la-Neuve, Belgium; R.C. = senior author; R.O. = W.R. = Dr. William Rains, Davis, University of California, CA, USA.

There were 34 minor fatty acid components (each less than 0.9 % of the total) detected and identified constituting 12.08% of total, and including hydroxy-substituted acids (class total of 1.81%), branch-chains in addition to the *iso*-16:0, and cyclopropane acids (0.54%) (Table 2).

TABLE 2. Cellular fatty acids and mean percentage of 20 strains of cyanobionts from *Azolla* spp.

| Fatty acids                                  |                  |        |             | Frequency<br>of<br>occurrence |
|--|------------------|--------|-------------|-------------------------------|
| Chemical class                               | ECL <sup>1</sup> | Mean % | Range       |                               |
| Saturated, even-carbon<br>straight chains:   |                  |        |             |                               |
| 8:0  | 8.0              | 0.04   | 0.00- 0.13  | 71                            |
| 10:0   | 10.0             | 0.03   | 0.00- 0.14  | 81                            |
| 12:0   | 12.0             | 0.29   | 0.07- 0.86  | 100                           |
| 14:0   | 14.0             | 0.98   | 0.33- 1.69  | 100                           |
| 16:0   | 16.0             | 34.83  | 30.29-39.53 | 100                           |
| 18:0   | 18.0             | 1.94   | 0.62- 4.58  | 100                           |
| 20:0   | 20.0             | 0.22   | 0.01- 0.91  | 100                           |
| Class A total                                |                  | 38.33  | 31.61-43.26 | 100                           |
| B. Saturated, odd-carbon<br>straight chains: |                  |        |             |                               |
| 9:0  | 9.0              | 0.03   | 0.00- 0.20  | 43                            |
| 11:0   | 11.0             | 0.01   | 0.00- 0.06  | 29                            |
| 13:0   | 13.0             | 0.03   | 0.00- 0.12  | 48                            |
| 15:0   | 15.0             | 0.44   | 0.09- 1.01  | 100                           |
| 17:0   | 17.0             | 0.29   | 0.06- 0.64  | 100                           |
| 19:0   | 19.0             | 0.12   | 0.00- 0.58  | 76                            |
| Class B total                                |                  | 0.91   | 0.51- 2.07  | 100                           |
| C. Unsaturated, straight<br>chain acids:     |                  |        |             |                               |
| 12:1   | 11.8             | 1.16   | 0.18- 6.14  | 100                           |
| 13:1   | 12.8             | 0.01   | 0.00- 0.07  | 29                            |
| 14:1 <i>cis</i> -7                           | 13.8             | 0.17   | 0.00- 0.48  | 66                            |
| 14:1 <i>cis</i> -9                           | 13.9             | 2.03   | 0.58- 3.51  | 100                           |
| 15:1   | 14.9             | 0.04   | 0.00- 0.32  | 20                            |
| 16:4(?) <sup>2</sup>                         | 15.2             | 0.18   | 0.00- 0.68  | 8                             |
| 16:? <sup>2</sup>                            | 15.5             | 1.35   | 0.00- 2.56  | 91                            |
| 16:4 <i>cis</i> -4                           | 15.55            | 1.04   | 0.00- 2.56  | 95                            |
| 16:3 <i>cis</i> -6                           | 15.6             | 2.35   | 0.00- 4.42  | 91                            |
| 16:1 <i>cis</i> -7                           | 15.75            | 1.04   | 0.00- 2.96  | 76                            |
| 16:1 <i>cis</i> -9                           | 15.8             | 5.30   | 3.31- 8.39  | 100                           |
| 16:1 <i>trans</i> -9                         | 15.85            | 0.49   | 0.00- 3.29  | 57                            |
| 16:1 <i>cis</i> -11                          | 15.9             | 1.96   | 0.00- 4.32  | 91                            |
| 16:1 <i>trans</i> -3                         | 15.95            | 0.73   | 0.00- 2.80  | 81                            |
| 18:3 <i>cis</i> -6                           | 17.2             | 0.75   | 0.39- 1.92  | 100                           |
| 18:4 <i>cis</i> -6                           | 17.5             | 0.20   | 0.00- 0.89  | 95                            |
| 18:2 <i>cis</i> -9                           | 17.7             | 9.45   | 5.48-20.00  | 100                           |

**TABLE 2. Continued** Cellular fatty acids and mean percentage of 20 strains of cyanobionts from *Azolla* spp.

| Fatty acids              |                  |        |             | Frequency<br>of<br>occurrence |
|--------------------------|------------------|--------|-------------|-------------------------------|
| Chemical class           | ECL <sup>1</sup> | Mean % | Range       |                               |
| 18:3 <i>cis</i> -9       | 17.75            | 17.88  | 8.76- 30.00 | 100                           |
| 18:1 <i>cis</i> -9       | 17.8             | 3.06   | 1.09- 5.11  | 100                           |
| 18:1 <i>trans</i> -9     | 17.85            | 2.03   | 0.00- 4.48  | 95                            |
| 20:4 <i>cis</i> -5       | 19.2             | 1.04   | 0.40- 2.52  | 100                           |
| 20:4 <i>cis</i> -8       | 19.4             | 0.31   | 0.01- 1.23  | 100                           |
| 20:2 <i>cis</i> -11      | 19.6             | 0.29   | 0.01- 0.65  | 100                           |
| Class C total            |                  | 53.04  | 44.63-58.51 | 100                           |
| D. Hydroxy-substituted:  |                  |        |             |                               |
| 2OH-10:0                 | 11.15            | 0.08   | 0.00- 0.37  | 67                            |
| 3OH-10:0                 | 11.4             | 0.20   | 0.04- 1.49  | 100                           |
| <i>iso</i> 3OH-11:0      | 12.1             | 0.13   | 0.01- 0.35  | 100                           |
| 2OH-12:0                 | 13.2             | 0.06   | 0.00- 0.28  | 48                            |
| 3OH-12:0                 | 13.5             | 0.03   | 0.00- 0.15  | 43                            |
| <i>iso</i> 3OH-14:0      | 15.1             | 0.63   | 0.00- 1.38  | 95                            |
| <i>iso</i> 3OH-15:0      | 16.1             | 0.03   | 0.00- 0.27  | 24                            |
| <i>iso</i> 3OH-17:0      | 18.1             | 0.25   | 0.01- 1.23  | 100                           |
| 2OH-17:0                 | 18.2             | 0.07   | 0.00- 0.38  | 43                            |
| 3OH-17:0                 | 18.4             | 0.33   | 0.14- 0.80  | 100                           |
| Class D total            |                  | 1.81   | 1.08- 2.67  | 100                           |
| E. Branched chain acids: |                  |        |             |                               |
| <i>iso</i> -11:0         | 10.6             | 0.01   | 0.00- 0.07  | 29                            |
| <i>iso</i> -13:0         | 12.6             | 0.09   | 0.00- 0.19  | 100                           |
| <i>iso</i> -14:0         | 13.6             | 0.08   | 0.00- 0.24  | 76                            |
| <i>iso</i> -15:0         | 14.6             | 0.05   | 0.00- 0.23  | 76                            |
| <i>anteiso</i> -15:0     | 14.7             | 0.06   | 0.00- 0.25  | 81                            |
| <i>iso</i> -16:0         | 15.6             | 2.09   | 0.01- 5.22  | 100                           |
| <i>iso</i> -17:0         | 16.6             | 0.41   | 0.17- 1.08  | 100                           |
| <i>anteiso</i> -17:0     | 16.7             | 0.23   | 0.05- 0.70  | 100                           |
| <i>iso</i> -19:0         | 18.6             | 0.06   | 0.00- 0.19  | 76                            |
| <i>anteiso</i> -19:0     | 18.7             | 0.10   | 0.00- 0.30  | 76                            |
| Class E total            |                  | 3.20   | 1.08- 4.68  | 100                           |
| F. Cyclopropane acids:   |                  |        |             |                               |
| cyclo-17:0               | 16.9             | 0.11   | 0.00- 0.29  | 81                            |
| cyclo-19:0               | 18.9             | 0.43   | 0.00- 1.30  | 76                            |
| Class F total            |                  | 0.54   | 0.00- 1.38  | 91                            |

**TABLE 2. Continued** Cellular fatty acids and mean percentage of 20 strains of cyanobionts from *Azolla* spp.

| Fatty acids                                |                  |        |            | Frequency of occurrence |
|--|------------------|--------|------------|-------------------------|
| Chemical class                             | ECL <sup>1</sup> | Mean % | Range      |                         |
| G. Unsaturated branch-chained fatty acids: |                  |        |            |                         |
| <i>iso</i> -15:1                           | 14.35            | 0.01   | 0.00- 0.05 | 29                      |
| <i>iso</i> -17:1                           | 16.4             | 0.30   | 0.13- 0.81 | 100                     |
| <i>iso</i> -18:1                           | 18.4             | 0.55   | 0.01- 3.70 | 91                      |
| Class G total                              |                  | 0.97   | 0.25- 4.54 | 100                     |

<sup>1</sup>- ECL = equivalent (carbon) chain length

<sup>2</sup> Unsaturation sites undetermined

Two fatty acids, 16:1 *trans*-3 and *iso*3-OH 12:0, were present in cyanobionts and have not been previously reported in *Anabaena* or *Nostoc*. Mean values for rank and similarity coefficients, respectively, for the *Azolla* isolates were 5.0 and 0.718 for *A. rubra*, 6.67 and 0.714 for *A. microphylla*, 9.33 and 0.688 for *A. caroliniana*, 6.67 and 0.681 for *A. nilotica*, 11.0 and 0.677 for *A. mexicana*, 14.67 and 0.655 for *A. pinnata*, and 19.33 and 0.612 for *A. filiculoides*. Based on analysis of rank and similarity coefficients, cyanobionts from *A. rubra* had fatty acid profiles statistically different from *A. pinnata* and *A. filiculoides*. Profiles of isolates from spp. *mexicana*, *nilotica*, *caroliniana* and *microphylla*, constituted an intermediate group.

**TABLE 3.** Rank analysis of *Azolla* cyanobionts, by host species, based on similarity coefficient(s) of individual fatty acid profiles.

| Strain | Host                  | (S) <sup>1</sup> | Mean + deviation <sup>2</sup> | Rank <sup>3</sup> | Mean rank <sup>3</sup> |
|--------|-----------------------|------------------|-------------------------------|-------------------|------------------------|
| C3     | <i>A. caroliniana</i> | 0.707            | 0.688 ± 0.017                 | 4                 | 9.33 ab                |
| C8     |                       | 0.685            |                               | 11                |                        |
| C2     |                       | 0.673            |                               | 13                |                        |
| M1     | <i>A. microphylla</i> | 0.757            | 0.714 ± 0.039                 | 1                 | 6.67 ab                |
| M4     |                       | 0.683            |                               | 7                 |                        |
| M2     |                       | 0.702            |                               | 12                |                        |
| X10    | <i>A. mexicana</i>    | 0.705            | 0.677 ± 0.677                 | 6                 | 11.00 ab               |
| X5     |                       | 0.692            |                               | 10                |                        |
| X1     |                       | 0.633            |                               | 17                |                        |

**TABLE 3.** Continued Rank analysis of *Azolla* cyanobionts, by host species, based on similarity coefficient(s) of individual fatty acid profiles.

| Strain          | Host                   | (S) <sup>1</sup> | Mean + deviation <sup>2</sup> | Rank <sup>3</sup> | Mean rank <sup>3</sup> |
|-----------------|------------------------|------------------|-------------------------------|-------------------|------------------------|
| N2              | <i>A. nilotica</i>     | 0.720            |                               | 3                 |                        |
| N1              |                        | 0.671            |                               | 14                |                        |
| N1 <sup>4</sup> |                        | 0.651            | 0.681 ± 0.035                 | 12                | 6.67 ab                |
| R3              | <i>A. rubra</i>        | 0.748            |                               | 4                 |                        |
| R4              |                        | 0.707            |                               | 10                |                        |
| R1              |                        | 0.701            | 0.718 ± 0.026                 | 15                | 5.00 a                 |
| P1              | <i>A. p. pinnata</i>   | 0.669            |                               | 15                |                        |
| I1              | <i>A. p. imbricata</i> | 0.693            |                               | 9                 |                        |
| I2              |                        | 0.604            | 0.655 ± 0.046                 | 20                | 14.67 b                |
| F12             | <i>A. filiculoides</i> | 0.622            |                               | 18                |                        |
| F10             |                        | 0.619            |                               | 19                |                        |
| F18             |                        | 0.594            | 0.612 ± 0.015                 | 21                | 19.33 b                |

<sup>1</sup> Coefficient based on percent proximity of individual fatty acid profile to the mean profile of all strains (as described in Table 1).

<sup>2</sup> Mean coefficient ± standard deviation for each host group.

<sup>3</sup> Ranking based on similarity coefficients. Means (in column) not followed by the same letter are significantly different at the 95% level of probability ( $p = 0.05$ ).

<sup>4</sup> Duplicate run.

## DISCUSSION

Twenty one isolates of cyanobacteria symbiotically associated with 7 different species of the aquatic water fern, *Azolla* (3 isolates per species), were extracted and analyzed for cellular fatty acid composition by gas-liquid chromatography-mass spectroscopy. Fatty acid profiles of cyanobionts contained variability beyond that explained by experimental or analytical procedures. Rank analysis of similarity coefficients suggest that profiles of isolates from *A. rubra* were statistically distinct from those of *A. pinnata* spp. and *A. filiculoides*.

The reasons for differences in fatty acid profiles may not necessarily be genetic. Physiological conditions and environmental factors can influence fatty acid composition of eukaryotic or prokaryotic cells. It is possible that light quality is significantly different in cavities of some species of *Azolla*, or that nutritional conditions may be different.

Regarding the possibility that the cyanobacteria of *A. filiculoides* and *A. pinnata* spp. may be genetically different from those of *A. rubra*, such conclusions are not supported totally with morphological data (unpublished data). Nevertheless, final judgement would depend in studies of larger *Azolla* cyanobiont populations and/or complete genetic data. In any event, this study was not

intended as a taxonomic analysis of *Azolla* cyanobionts, but as a description of the variability found in fatty acid profiles of *Azolla* cyanobionts, as may be influenced by species.

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Reprint requests to Dr. Rodulio Caudales